Protamine Inhibits Capillary Formation in Growing Rat Hearts
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SUMMARY. Various indices of capillary supply to the rat heart were studied in neonatal rats injected for 2 or 4 weeks with protamine sulfate in saline (subcutaneously, 60 mg/kg body weight, 2 times/day). Cardiac capillarization was evaluated not only by traditional indices for the capillary supply, such as mean capillary density and myocyte-to-capillary ratio, but also by a more advanced morphometric method of capillary domains. This method allows the estimation of both the average radius of the Krogh tissue cylinder and its variability, which reflects the heterogeneity of capillary spacing found to be an independent morphological determinant of oxygen diffusion in the tissue. The results were evaluated with respect to regional differences (subendocardial vs. middle section), age differences, and the effect of protamine. No regional differences in capillary supply were found in this experimental situation. Hearts from older rats had significantly decreased capillary supply, expressed as lower capillary density, larger capillary domains, and greater radius of the tissue cylinder. On the other hand, the heterogeneity of capillary spacing decreased significantly with age. Protamine-injected animals, when compared to their control littermates, had a significantly higher cell-to-capillary ratio, lower capillary density, larger capillary domains, greater radius of the tissue cylinder, and larger variability in capillary spacing. Thus, protamine was effective in impeding rapid capillary growth in the hearts from rats in the early postnatal period. Close to half of all the existing capillaries in the adult rat hearts are formed during the first 3-4 postnatal weeks. (Circ Res 57: 393-398, 1985)

MECHANISMS regulating capillary growth in mammalian hearts are poorly understood. Most of the capillary growth takes place in the early postnatal period. Later on, capillary proliferation decreases and the adult stage is characterized by a constant number of capillaries and myocytes (Rakusan, 1984). The reasons for this slow-down and eventual cessation of capillary growth are not known. Similarly, pathological growth of the heart leading to cardiac hypertrophy is characterized by rarefaction of the capillary net due to little or no capillary growth in this situation (Rakusan, 1971; Hudlicka, 1984). There are, however, occasional reports of capillary proliferation in adult mammalian hearts induced by exercise, propranolol, or dipyridamole treatment and long-term bradycardial pacing (e.g., Tomanek, 1970; Tašgal and Williams, 1981; Tornling, 1982; Anversa et al., 1983; Hudlicka et al., 1984; Ljungquist et al., 1984). Once again, the mechanisms leading to this renewal of capillary formation are not known.

Angiogenesis is an important biological issue. Whereas stimulation of vascular growth can be beneficial in the hypertrophic heart or for wound healing, prevention or limitation of vascular growth is of practical importance in ophthalmology and in oncology. For a review of earlier literature on angiogenesis, see Schoeff (1963); more recent literature is summarized by Hudlicka (1984). In 1975, Kessler and coworkers reported that mast cells assemble at a tumor site before the ingrowth of new capillaries, and proposed that they may play a role in tumor angiogenesis. This was followed by a suggestion that mast cells release a factor that stimulates migration of capillary endothelial cells. Azizkhan and coworkers (1980) investigated the effect of isolated mast cell products on bovine capillary endothelial cell migration in vitro and demonstrated that heparin is the mast cell factor responsible for capillary endothelial cell migration. This effect was blocked by protamine. Subsequent reports indicated that protamine also prevents the neovascularization induced by an inflammatory agent or by an immune reaction. According to Taylor and Folkman (1982), it can also inhibit tumor angiogenesis and subsequent tumor growth. However, protamine has no effect on established capillaries that are not proliferating. On the other hand, heparin can enhance the intensity of angiogenesis induced by tumor extract in vivo, but it cannot by itself initiate angiogenesis.

In this presentation, we report evidence that protamine has a similar inhibitory effect on rapid capillary formation in rat hearts during the early postnatal period.

Methods
Young male Sprague-Dawley rats were used in the experiments. To secure uniform growth of animals, we limited the number of pups used per litter to eight. From
day 10, experimental animals were injected every 12 hours subcutaneously with protamine sulfate in saline (60 mg/kg of body weight). The stock solution was prepared by the method described by Taylor and Folkman (1982). The dosage of protamine used in our experiments on newborn rats is the same as that used by Taylor and Folkman (1982) for successful suppression of lung metastasis on mice of similar body weight. For the same effect on adult rats, these authors used a double dosage of protamine. Control animals received aliquot injections of saline at the same times. In the first set of experiments, the injections were given for 2 weeks, and the animals were killed on the following day (project 1 contained nine control and nine experimental animals). In the second set of experiments, the animals were injected twice a day for 28 days and killed within a week after the last injection (project 2: six control and six experimental rats).

At the time of sacrifice, the animals were anesthetized with sodium pentobarbital and their hearts were perfused in situ with heparinized saline and fixed with a mixture of 1.5% glutaraldehyde buffered to pH 7.4 with phosphate buffer. The perfusion pressure was 80–90 mm Hg; the perfusion time was 20 minutes. Subsequently, the hearts were removed, and the ventricles were separated from the atria and weighed. The right ventricular free walls were discarded and the left ventricular free walls, together with septa, were cut at one-third of the distance from the base to the apex of the heart. After fixation and dehydration in alcohol, the samples were embedded in Sorval embedding medium (methacrylate). Sections 1 μm thick were stained by Avallone’s modification of Jones’ silver methenamine method for staining basement membranes (Sorvall, 1979).

Photomicrographs were taken of the subendocardial and middle regions of the left ventricular free wall. From both cardiac regions, five to seven photomicrographs were taken, each having an area of 28,500 μm². First, traditional indices of cardiac capillarization were determined: average capillary density (number of capillaries/mm²) and myocyte-to-capillary ratio. From the capillary density, it is possible to compute the average radius of the Krogh tissue cylinder. Tissue oxygenation, however, is influenced not only by the mean values of the Krogh cylinder, but also by its variability, i.e., by the heterogeneity of the capillary spacing (Turek and Rakusan, 1981). Variability of the tissue cylinder radius was estimated from the recently proposed method of capillary domains (Hoofd et al., 1985). The principle of this method is to assign a "surrounding area" to each capillary on the tissue cross-section.

### Table 1

<table>
<thead>
<tr>
<th>Basic Data</th>
<th>Project 1</th>
<th>Project 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Controls</td>
<td>Experimental</td>
</tr>
<tr>
<td>Body wt (g)</td>
<td>54.4 ± 7.3</td>
<td>48.6 ± 7.4</td>
</tr>
<tr>
<td>Left ventricle and septum (mg)</td>
<td>224 ± 28</td>
<td>188 ± 39*</td>
</tr>
<tr>
<td>mg/g body wt</td>
<td>4.2 ± 0.4</td>
<td>3.9 ± 0.5</td>
</tr>
<tr>
<td>% dry wt (dry wt/wet wt × 100)</td>
<td>20.1 ± 1.1</td>
<td>22.3 ± 1.8</td>
</tr>
<tr>
<td>No. of rats</td>
<td>9</td>
<td>9</td>
</tr>
</tbody>
</table>
rate of cardiac growth was not specifically influenced by protamine injection, as the heart weight:body weight ratio was the same in both experimental groups.

Data concerning the capillary supply of the heart are summarized in Figure 2 and Table 2. Average capillary density, as depicted on Figure 2, and myocyte-to-capillary ratio, included in Table 2, are traditional indicators of the capillary supply to the heart. These parameters were complemented by measurements of the capillary domains with subsequent calculations of the radii of the Krogh tissue cylinders (R), as described in Methods. Variability of these parameters, expressed as log SD, served as an index of the heterogeneity of capillary spacing. These data were analyzed by ANOVA with respect to regional differences (subendocardial vs. middle section), age differences (younger animals from project 1 vs. older animals from project 2), and the effect of protamine injections (experimental animals vs. controls).

Results of all the above parameters were essentially the same in both middle and subendocardial regions. In addition, the regional effect did not interact significantly with the remaining analysis. As expected, older animals had a significantly decreased capillary supply, expressed as lower capillary density, a larger capillary domain, and a greater R (P < 0.01). Also, the cell-to-capillary ratio was lower in older animals (P < 0.05). Finally, heterogeneity of capillary spacing (log SD) decreased significantly with age (P < 0.01).

Protamine injections definitely interfered with normal capillary growth in this developmental pe-
period. Analysis of variance revealed an overall decrease in capillary density in protamine-injected animals, associated with a higher cell-to-capillary ratio, a larger capillary domain, and a greater \( R \) (all significant at \( P < 0.01 \)). At the same time, heterogeneity of capillary spacing (log SD) in protamine-injected animals was significantly larger. The difference in log SD was also statistically significant in younger animals evaluated as a single group but not in the older group. Finally, there was a significant interaction at \( P < 0.05 \) between the effects of age and protamine injection on the capillary density, size of capillary domains, and radii of Krogh tissue cylinders. This simply means that, in older animals which were injected with protamine for a longer period of time, the effect of protamine on various indices of myocardial supply was more pronounced. Finally, Table 2 also contains statistical significance of differences between control and experimental animals when morphometric results in each cardiac region of both projects were compared separately.

**Discussion**

We found no differences in any of the morphological indicators of the capillary supply between midwall and subendocardial regions of hearts from normal, growing rats. This is in agreement with most reports concerning regional differences in normal adult rat hearts (e.g., Anversa et al., 1978; Lund and Tomanek, 1978; Rakusan et al., 1980). Occasionally, the subepicardial region, not examined in the present experiments, has been reported to have a higher capillary density (Gerdes et al., 1979).

Similarly, our finding of decreasing relative capillary supply in hearts from older animals is in agreement with previous developmental studies in which the rapid capillary growth terminated during the weaning period and subsequently capillary density decreased with increasing age and body weight (for a review, see Rakusan, 1984). Age-related changes in the heterogeneity of the capillary spacing in the rat heart has been examined here for the first time. We found that smaller log SD was associated with an increased radius of the Krogh cylinder in the hearts from older animals. The diminished heterogeneity, which improved the morphological conditions for oxygen supply, is expected to compensate for the \( P_{O_2} \) decreasing effect of the increased radius of the tissue cylinder (Turek and Rakusan, 1981).

However, the observed changes are relatively small, and in spite of statistical significance at \( P < 0.01 \), their biological importance remains uncertain. Nevertheless, they indicate the developmental trends in the rat heart.

In each animal, heterogeneity of the capillary spacing was also estimated by an alternative method that used the distances between capillaries and regularly distributed grid points [our adaptation of the method of the closest neighbor (Kayar et al., 1982; Hoofd et al., 1985)]. For the sake of brevity, we did not include those data in the present article. However, it is reassuring to note that all the differences in log SD reported above were also independently validated by this alternative method.

Our major aim was to study the effect of protamine on the capillary growth in neonatal rat heart. All the results presented seem to support our hypothesis that this treatment effectively inhibits capillary growth in the hearts from rats during early postnatal development. In protamine-treated animals, capillary density was significantly lower and the amount of tissue supplied by a single capillary was larger (larger capillary domain and greater tissue cylinder radius). This was accompanied by increased heterogeneity of capillary spacing. All these differences were more pronounced after longer treatment with protamine (project 2). The myocyte-to-capillary ratio did not change with age in protamine-treated rats, although it decreased significantly in control animals. Since the total number of myocytes in these hearts has already attained a constant value, this indicates no capillary formation in protamine-treated hearts, in contrast to a still active capillary growth in the control group.

All the above results seem to indicate that protamine impedes capillary formation in neonatal hearts. How many capillaries are actually formed in normal neonatal rat hearts? We can estimate that close to 6 \( \times 10^6 \) capillaries are actually formed in normal neonatal rat hearts. We can estimate that close to half of all the capillaries present in the normal adult rat heart originate during the first 3 postnatal weeks. This stems from the following appraisal: left ventricles from adult rats contain approximately 30 million myocytes (Rakusan et al., 1981). The average range of coronary capillary lengths is 500–600 \( \mu \)m, which is approximately 5 times the length of cardiac myocytes (Ludwig, 1971; Korecky and Rakusan, 1978). With approximately equal numbers of myocytes and capillaries in the cross-sections of the adult hearts, the estimate for the total number of capillaries would be around 6 \( \times 10^6 \); this is taken as 100% of adult values in subsequent computations. In the hearts

**Table 3**

<table>
<thead>
<tr>
<th>No. of myocytes</th>
<th>Myocyte:capillary ratio</th>
<th>No. of capillaries</th>
<th>% of adult values</th>
</tr>
</thead>
<tbody>
<tr>
<td>At birth</td>
<td>2 ( \times 10^7 )</td>
<td>16.4</td>
<td>0.4 ( \times 10^9 )</td>
</tr>
<tr>
<td>Weaning</td>
<td>3 ( \times 10^7 )</td>
<td>1.7</td>
<td>3.6 ( \times 10^9 )</td>
</tr>
<tr>
<td>Adult</td>
<td>3 ( \times 10^7 )</td>
<td>1.0</td>
<td>6.0 ( \times 10^9 )</td>
</tr>
</tbody>
</table>
from rats in the weaning period, the total number of cardiac myocytes is already the same as in adult hearts, but the myocyte-to-capillary ratio is only 1.7 (Rakusan 1984). Thus, the estimated total number of capillaries in the left ventricle from these rats would be $3.6 \times 10^6$, which is only 60% of the adult values. On the other hand, at birth, the total number of cardiac myocytes in the left ventricle has only reached approximately $2 \times 10^6$, which is a mere 15% of the adult values (see Table 3). The purpose of this exercise was to demonstrate the impressive rate of capillary formation in the early postnatal period. Close to half of all adult capillaries were formed during the relatively short period of 3–4 weeks. Similarly, Olivetti and coworkers (1980) reported that during the first 10 postnatal days, capillaries grow two to three times more rapidly than the myocardial mass.

Our results demonstrate that, in addition to the experimental situations described in the introduction, protamine is also effective in impeding the rapid capillary growth of the coronary capillaries in the early postnatal period. The mechanism by which protamine inhibits capillarogenesis is not clear. Whereas one cannot exclude the direct effect of this compound on proliferating capillary endothelial cells, a more probable explanation lies in its blockage of the effect of heparin, locally released by mast cells. Mast cells are usually closely associated with the terminal vascular bed, and their contribution to vascular growth during injury has already been proposed by Smith (1961) and Schoefl (1963). Regrettably, we are not aware of any study dealing with mast cells in mammalian hearts during the early postnatal period. In addition, although heparin itself was found to promote angiogenesis, its simultaneous application with cortisone resulted in inhibition of this phenomenon as recently reported by Folkman and coworkers (1983). Further research involving the heparin-protamine hypothesis in regulation of capillary growth is therefore strongly indicated.

INDEX TERMS: Terminal vascular bed • Capillary formation • Cardiac development • Heparin
Protamine inhibits capillary formation in growing rat hearts.

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